

Distinct versus redundant properties among members of the INK4 family of cyclin-dependent kinase inhibitors

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Received 12 February 2000

Edited by Julio Celis

Abstract p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d} comprise a family of cyclin-dependent kinase inhibitors and tumor suppressors. We report that the INK4 proteins share the ability to arrest cells in G1, and interact with CDK4 or CDK6 with similar avidity. In contrast, only p18 and particularly p19 are phosphorylated in vivo, and each of the human INK4 proteins shows unique expression patterns dependent on cell and tissue type, and differentiation stage. Thus, the INK4 proteins harbor redundant as well as non-overlapping properties, suggesting distinct regulatory modes, and diverse roles for the individual INK4 family members in cell cycle control, cellular differentiation, and multistep oncogenesis.

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Key words: Cell cycle; p16^{INK4a}; p15^{INK4b}; p18^{INK4c}; p19^{INK4d}; Cyclin-dependent kinases 4 and 6

1. Introduction

The cyclin-dependent kinases (CDKs) are key regulators of the cell cycle, and as such their activities must be tightly controlled [1,2]. In mammalian cells, the CDKs are negatively regulated by proteins of two families of CDK inhibitors (CKIs), the INK4 and Cip/Kip families [1–4]. CKIs of the INK4 family, p16^{INK4a} (p16), p15^{INK4b} (p15), p18^{INK4c} (p18), and p19^{INK4d} (p19), specifically bind and inhibit CDK4 and CDK6, the partner kinases of the D-type cyclins strongly implicated in phosphorylation of the retinoblastoma tumor suppressor protein (pRB) and thereby in G1/S control [3–5]. On the other hand, CKIs of the Cip/Kip family, p21, p27, and p57, inhibit a broader spectrum of CDKs including cyclin D/CDK4(6), cyclin E/CDK2, and cyclin A/CDK2 [1–4]. The four proteins of the INK4 family share a similar structure dominated by several ankyrin repeats, and the corresponding genes contain an intron which interrupts the coding sequence at the same position, indicating that they evolved from a common ancestor [4,6,7]. In terms of biological functions, different CKIs appear to participate in regulation and coordination of cell cycle events following mitogenic stimulation, DNA damage, mitogen deprivation, or changes in cell–cell or cell–matrix interactions, and they have also been implicated in inducing terminal differentiation and cellular aging or senescence [3–7]. Apart from their physiological roles, the CKIs are

commonly lost or inactivated by mutations in diverse types of cancer, and they represent established or candidate tumor suppressors. Thus, inactivation of p16 through gene deletions, point mutations or transcriptional silencing by promoter methylation is among the most frequent defects contributing to oncogenesis [4–7] and, although much less extensive, there is also evidence for abnormalities of the other INK4 proteins in some tumors [6–9].

Given their emerging roles in fundamental physiological as well as pathological processes, the biochemical similarities and evolutionary homology of the INK4 family members raise an important question of redundancy versus non-overlapping features of p16, p15, p18 and p19. Despite the recent efforts in many laboratories, the relevant data remains scattered and partly conflicting, and further work is needed to clarify this complex issue. For instance, it remains a matter of debate whether the individual INK4 proteins favor CDK6 over CDK4 as their target [6,10,11], and while transcriptional regulation of the INK4 genes is well established, it is unclear whether the INK4 proteins are controlled also by posttranslational modifications, at the level of regulation which is commonly employed to control abundance or function of other cell cycle regulators including CKIs of the Cip/Kip family [4]. Differential expression of the INK4 genes in different mouse tissues has been documented by mRNA analyses [12–15], yet analogous studies in humans are lacking, and particularly data at the protein level, in terms of tissue or cell type specificity and abundance, are virtually missing for any species. In an attempt to address some of these open questions, we have used a range of biochemical and immunochemical approaches to examine the degree of redundancy within the human INK4 family of cell cycle inhibitors, and our results are presented in this report.

2. Materials and methods

2.1. Plasmids and antibodies

Human cDNAs for p15, p16, p18 and p19 were subcloned into the vector pXmyc for expression in mammalian cells. Monoclonal antibodies DCS-118 and DCS-100 were produced upon immunization of young female BALB/c mice with bacterially produced human His-tagged, full-length p18, and GST-tagged full-length p19, respectively, using established procedures [16,17]. Other antibodies were: mouse monoclonal anti-CDK6 (DCS 130.1) [17], anti-CDK4 (DCS 31.1) [17], anti-p21 (DCS-60) [16], anti-p15 (Ab-4, NeoMarkers), anti-p16 (DCS-50) [18], anti-p16 (Pierce), anti-p16 (Ab-2, NeoMarkers), anti-myc-epitope (9E10, provided by G. Evan), polyclonal rabbit antisera to CDK4 (sc601, Santa Cruz), p18 (M-168, Santa Cruz), p15 (C-20, Santa Cruz), and secondary rabbit anti-mouse IgG-HRP (Dako) and goat anti-rabbit IgG-HRP (Vector).

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2.2. Cell culture, transfection, and flow cytometry

Human tumor-derived cell lines U-2-OS, Saos-2, and SK-UT-1 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics. Human teratocarcinoma cells NT2/D1 were cultured and induced to differentiate exactly as described [9,19]. Transient transfections to express the INK4 genes in U-2-OS cells were performed using the previously described calcium phosphate precipitate method [18]. Proteins were allowed to be expressed for 0–36 h after washing off the precipitate, and the flow cytometry analyses of cell cycle distribution determined as described [18].

2.3. Immunochemical and immunostaining methods

Procedures for immunoprecipitation, gel electrophoresis, and immunoblotting were described previously, as were the techniques for immunofluorescence staining of cells cultured on coverslips [9,16–20]. The human tissues were from the tissue bank of the Institute of Cancer Biology, Copenhagen, or obtained commercially as the Checkboard Multi-Tissue Blocks (Dako) containing sections from a wide spectrum of normal human tissues, formalin-fixed, paraffin-embedded, and mounted on poly-L-lysine-coated slides. The sensitive immunoperoxidase staining using the Vectastain Elite kit (Vector) was performed as described previously [9,16,17]. Immunoprecipitations were made as in [17], with the antibody 9E10 coupled to protein G-Sepharose and 500 µg protein extract. Immunoprecipitated proteins were separated on 13% polyacrylamide gel electrophoresis (PAGE), transferred onto nitrocellulose membranes by semidry blotting, the membranes probed with the relevant antibodies and the signal visualized by enhanced chemiluminescence, using either the standard or the SuperSignal ULTRA substrate (Pierce).

2.4. Production and purification of the INK4 fusion proteins

Human p15, p16, p18 and p19 cDNAs were cloned into the pGex20 T vector and expressed in BL21 *Escherichia coli* and purified as GST fusion proteins according to the manufacturer's instructions (Pharmacia). The BL21 cells were transformed and the fusion proteins induced overnight by IPTG (final concentration 0.1 mM). Bacterial pellets were collected and resuspended in ELB buffer (50 mM HEPES, pH 7.5, 250 mM sodium chloride, 5 mM EDTA, 0.1% NP-40, 1 mM dithiothreitol, 2.5 µg/ml leupeptin, 2 µg/ml aprotinin, 5 µg/ml phenylmethylsulfonyl fluoride), lysed by sonication and the extracts cleared by centrifugation. The supernatant was added to GST beads and incubated end-over-end for 30 min, at 4°C. The beads were washed five times in ELB buffer and the fusion protein eluted with 50 mM Tris-HCl (pH 8), 10 mM reduced glutathione. Eluted proteins were dialyzed against 10 mM Tris, pH 8. The protein concentration was determined by the Bradford protein assay (Bio-Rad) and the purity was estimated by SDS-PAGE and Coomassie staining.

2.5. Expression of CDK4 and CDK6 in insect cells, and in vitro competition assay

Baculoviruses expressing human CDK4 and CDK6 were amplified in Sf9 insect cells, and the High Five Insect cells were used to produce the protein [20]. The protein concentration in the insect cell lysates was estimated by the Bradford assay, and the lysates used for the in vitro competition assay [20] to measure the ability of the different INK4 proteins to interact with CDK4 or CDK6. The assay was performed in 96-well flat-bottomed microtiter plates (Nunc) coated with 40 nM GST-p16 diluted in coating buffer (final volume 100 µl). Unspecific sites were blocked by incubation with 5% dry powder milk for 1 h. The plates were washed, and serial dilutions of the INK4 proteins were conducted in the microtiter plate (concentration range 0.01–1 µM), followed by addition of the insect cell extract with either CDK4 or CDK6 (20 µg CDK4 extract and 50 µg CDK6 extract per well), in a final volume of 100 µl. After incubation for 1 h at 37°C, the plates were washed for 30 min, and incubated with either anti-CDK4 or anti-CDK6 antibodies for 2 h at 37°C, followed by washing and incubation with secondary antibodies, to detect the CDK bound to the p16 protein coating the plate. The assay was developed using the TMB⁺ solution as a chromogen (Kem-Tec, Denmark), and the color reaction was measured at 450 nm. The buffers used in the assay were as described [21].

The GST-p16 concentration required to saturate the microtiter plate, and the amounts of CDK4- and CDK6-containing insect cell extracts required to saturate the GST-p16-coated plate were estimated in preliminary titration experiments to set up the assay in a linear

range (data not shown). Each experiment was performed in triplicate, and repeated 3–4 times with very similar results.

2.6. Phosphopeptide mapping and phosphoamino acid analysis

Plasmids of interest were transfected into 25% confluent U-2-OS cells and the expression allowed for 12 h after washing off the precipitate. Cells were subsequently starved in phosphate-free DMEM for 30 min and then incubated in DMEM containing 2 mCi/ml [³²P]orthophosphate (Amersham) for 4 h on a rocking table at 37°C. Cells were washed in ice-cold phosphate-buffered saline and scraped and lysed in the extraction buffer. After centrifugation at 14000×g, the supernatant was precleared on protein G-Sepharose beads with DNase and RNase A (20 U of each) for 30 min at 4°C. Immunoprecipitation was performed as described above with the monoclonal antibody 9E10, with three additional washes. Eluted proteins were separated on 13% SDS-PAGE (Hoeffer, 20 cm length gel system) and in vitro translated myc-tagged p19 was used as a positional marker. Proteins were transferred onto nitrocellulose membrane and radioactive signal detected by autoradiography or on a phosphor-image screen (Molecular Dynamics). Bands of interest were excised and processed for tryptic cleavage, phosphopeptide mapping and phosphoamino acid analyses as described [21].

3. Results and discussion

3.1. Binding of INK4 proteins to CDK4 and CDK6, and G1 arrest

The four INK4 proteins share the ability to bind to CDK4 and CDK6, and inhibit the kinase activity of the cyclin D-CDK4(6) complexes [3–5], yet partly conflicting reports indicate that some of the INK4 proteins may favor CDK6 over CDK4 as their target, implying some functional differences among the various combinations of INK4-CDK interactions, and thus possibly differential impacts on cell cycle inhibition [6,10–12]. Factors potentially complicating any generalizations of such studies include the use of different antibodies specific for the individual INK4 or CDK proteins, and diverse cell lines with a considerably higher expression of CDK6 than CDK4, or with different levels of the (partly still elusive) assembly factors which facilitate the complex formation between CDK4(6) and the D-type cyclins [4,22]. To eliminate such variables, we transfected Myc-tagged p15, p16, p18 and p19 into parallel cultures of human U-2-OS cells which express wild-type pRB, all three D-type cyclins, and comparable levels of the CDK4 and CDK6 proteins, and examined the INK4-CDK4(6) complexes immunoprecipitated with the same antibody against the Myc epitope at 0, 2.5, 5, 8, 12, 16, and 24 h after removing the DNA calcium phosphate precipitate from the culture medium. The combined immunoprecipitation and immunoblotting experiments showed comparable ratios of the CDK4 and CDK6 kinases bound to each of the four INK4 proteins during these time course experiments, and a representative example of the data obtained at 5 and 16 h is shown in Fig. 1A. The same cellular model was also used to assess the cell cycle inhibitory properties of the ectopically expressed individual INK4 family members, and the flow cytometry profiles of the transfected cells showed a very similar, pronounced G1 arrest in every case (Fig. 1B). On the other hand, none of the four INK4 proteins showed any cell cycle inhibition upon transfection into the pRB-negative human sarcoma cell line Saos-2 (data not shown), consistent with the concept that the cyclin D-dependent kinases are required for cell cycle progression only in cells expressing wild-type pRB, a key substrate of cyclin D/CDK4(6) in G1 [4,5].

To directly compare the relative binding affinities of the

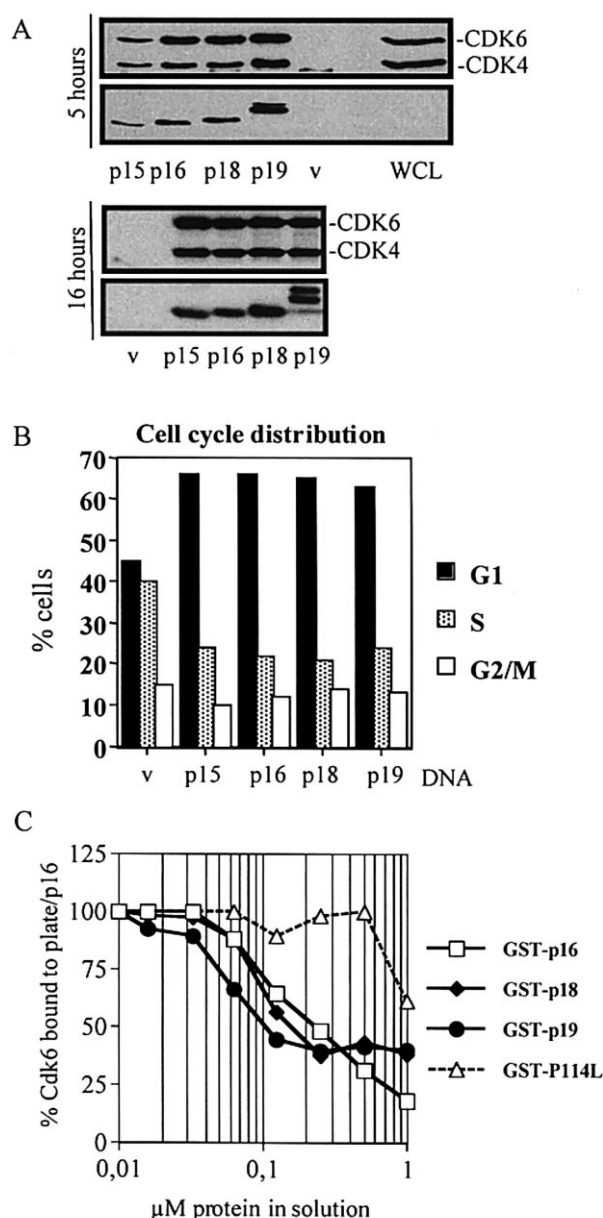


Fig. 1. Binding to CDK4 and CDK6, and induction of G1 arrest by human INK4 proteins. A: Myc-tagged p15, p16, p18, and p19 were ectopically expressed in U-2-OS cells, the INK4 proteins immunoprecipitated from cell extracts at indicated time points after removal of calcium phosphate precipitate via the 9E10 antibody to myc, and the complexes analyzed by immunoblotting for the presence of CDK4 and CDK6 (using a mix of DCS-31 and DCS-130 antibodies) and the tagged INK4 proteins (using 9E10); v=empty vector control; WCL=whole cell lysate of untransfected cells. B: A graph of a typical flow cytometry evaluation of the cell cycle effects of the myc-tagged INK4 proteins ectopically expressed in exponentially growing U-2-OS cells, compared to cells transfected with empty vector (v). C: Example of the plate binding assay, with increasing concentrations of soluble GST-p16, -p18, and -p19 competing for CDK6 with the plate-immobilized GST-p16.

individual INK4 proteins towards CDK4 and CDK6, we produced these human kinases in insect cells using the baculovirus expression system, and employed an *in vitro* binding assay [20] to assess the ability of purified recombinant human INK4 proteins to compete for CDK4(6) binding with solid phase-bound p16. The specificity of the assay was documented in

repeated experiments in which soluble wild-type p16, but not melanoma-associated mutant p16 (P114L) [18], efficiently prevented the binding of CDK4 or CDK6 to wild-type p16 immobilized on the plate (see Fig. 1C for an example). The P114L mutant p16 showed significant binding to CDK4(6) only at the highest concentration used (1 μM), consistent with previous data that this and other tumor-associated missense mutants of p16 are defective but not entirely devoid of the ability to bind and inhibit the cyclin D-dependent kinases [4,18]. Most significantly, wild-type versions of the other members of the INK4 family were able to compete for binding to CDK4(6) in a manner indistinguishable from the wild-type p16 used in parallel (Fig. 1C, and data not shown). Collectively, these immunoprecipitation and binding competition data are fully consistent with the notion that the INK4 proteins bind CDK4 and CDK6 with similar affinity, and that there are no major differences among the INK4 family members in their avidity towards the CDK4(6) partners, or the ability to impose a G1 arrest in RB-positive cells.

3.2. Differential phosphorylation of INK4 proteins *in vivo*

Phosphorylation and dephosphorylation events regulate function, subcellular localization, and turnover of diverse cell cycle regulatory proteins including the CDK inhibitors of the Cip/Kip family [1–4]. On the other hand, there have been no reports of phosphorylation of any of the INK4 proteins, despite intensive characterization of at least the most studied member, the p16 tumor suppressor [6,7]. To see whether any of the INK4 proteins is phosphorylated in human cells, wild-type p15, p16, p18 and p19 were transiently expressed to similar levels in exponentially growing U-2-OS cells, the cells were labeled with radioactive orthophosphate, and the immunoprecipitated INK4 proteins examined by gel electrophoresis and autoradiography and/or phosphorimage analysis. Unexpectedly, there was a clear distinction among the members of the family with respect to their phosphory-

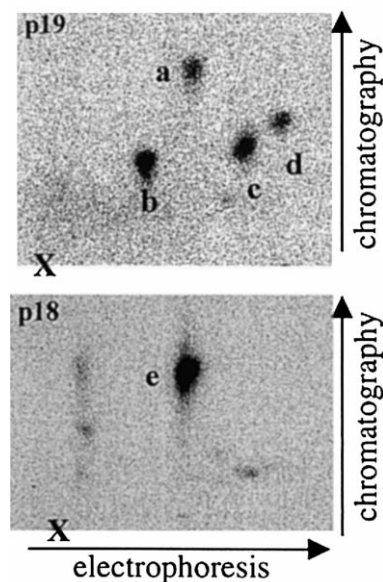


Fig. 2. Two-dimensional phosphopeptide maps of human p19 and p18 phosphorylated *in vivo*. X indicates the sample application spot; a–d: the four different phosphorylated peptides reproducibly found in tryptic digests of p19; e: the only tryptic phosphopeptide detected in p18.

lation. Whereas no ^{32}P labeling of p15 or p16 was found in repeated experiments, there was detectable phosphorylation in p18, and an even stronger signal in p19. Phosphoamino acid analysis of the proteins labeled *in vivo* revealed the presence of phosphoserine in p18, while p19 was phosphorylated on both serine and threonine residues. As can be seen from the representative examples of two-dimensional phosphopeptide maps (Fig. 2), tryptic digestion of p19 reproducibly resulted in four labeled spots termed here a–d, in contrast to p18 which appears to be labeled in only one peptide (Fig. 2). These experiments identified a previously unrecognized post-translational modification of a subset of the INK4 family members, and we are currently attempting to map the phosphorylation sites as a prerequisite for elucidating the biological significance of this phenomenon. Our initial cycle sequencing and site-directed mutagenesis experiments have so far identified serines 66 and 76 of human p19 as the residues phosphorylated in spots b and c (Fig. 2). Whereas spot b represents a peptide phosphorylated on both Ser 66 and 76, spot c corresponds to the same tryptic peptide but phosphorylated on Ser 76 only. The serines 66 and 76 are located on the surface of p19 as judged from its three-dimensional structure [23], and they are conserved in mouse p19, but not in the other three human INK4 proteins [6]. These results suggest why the cellular kinases which target p19 are unable to modify the other, closely related INK4 proteins, and the occurrence and complexity of phosphorylation separates p19 from the rest of the family. Such a specific series of phosphorylation events on phylogenetically conserved residues points to a candidate novel mechanism to control some aspect(s) of p19 function, a feature unique in the INK4 family, or at least distinct from p15 and p16.

3.3. Expression patterns of the INK4 proteins in human tissues

One feature that distinguishes the individual INK4 genes appears to be their distinct transcriptional control, in that their promoters respond differentially to diverse stimuli. In-

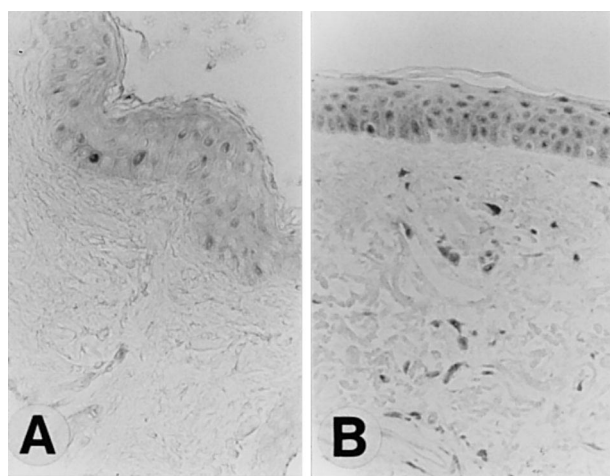


Fig. 3. Immunohistochemical detection of endogenous p18 and p16 in normal human skin. A: Immunoperoxidase staining with antibody against p16 (Pierce) is mainly restricted to some keratinocytes of the first suprabasal layer, and only rare stromal elements. B: Staining with antibody DCS-118 shows predominantly nuclear positivity of p18 in the majority of epidermal keratinocytes including several suprabasal layers, and numerous stromal cells. Magnification $\times 200$.

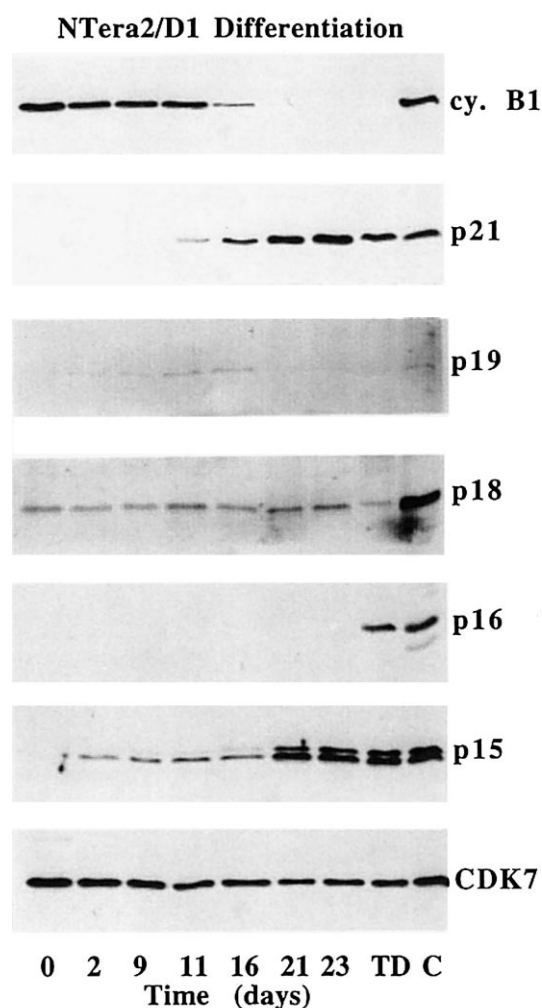


Fig. 4. Immunoblots of human NT2/D1 cells during retinoid acid-induced differentiation. Time 0 represents exponentially growing cells; TD are the enriched neuron-like 'terminally differentiated' cells 42 days after induction. Control cell lines (C) included U-2-OS for cyclin B1, p21, SK-UT-1 for p15, p16, p18, p19, and CDK7 (a stable protein used as a loading control). Blots probed with the indicated antibodies were developed using the standard chemiluminescence method, except for p15, p18, and p19 for which the SuperSignal ULTRA substrate (Pierce) was used to enhance the signal.

duction of p15 in response to transforming growth factor β , or upregulation of p16 with increasing population doublings or following some oncogenic stimuli are among examples of such differential transcriptional regulation (reviewed in [6,7]). Apart from studies with cultured cellular models, data from analyses of tissues *in situ*, so far largely limited to some mouse tissues and stages of development, suggest that the individual INK4 genes may be expressed in a stage- or cell type-restricted manner [12–15,24,25]. To test this attractive hypothesis in the human system where no comparative analysis of the INK4 proteins is presently available, we examined abundance and tissue type specificity of p16, p15, p18 and p19 immunohistochemically on a wide spectrum of human tissues using paraffin-embedded multi-tissue arrays. Interpretation of the immunostaining patterns by an experienced histopathologist allowed us to draw the following conclusions. In adult human tissues, the INK4 protein most widely expressed was p18,

followed by p15, p16, and p19 being the most restricted one. Thus, p18 was clearly detectable in all tissues examined, including diverse muscle, lymphoid, epithelial, neural and endocrine tissues, endothelium, and stromal cells, while p19 was largely negative, with only restricted cell types such as occasional lymphoid and rare glandular epithelial cells, seminiferous tubules, and adrenal gland cells showing detectable p19 protein. Unlike p18 which was expressed in an almost homogeneous pattern in many tissues, the staining signals for p15 and p16 were very variable from cell to cell, with the exception of endothelium and some simple (glandular) epithelia which were virtually homogeneously positive for p15. An example of the differential staining patterns is shown for p16 and p18 in the epidermis, with p18 clearly more widely expressed in both the stratified squamous epithelium and the stroma (Fig. 3A,B). Compared to available data on expression of the INK4 genes in mice, our results suggest a considerably narrower spectrum of tissues with detectable p19, and a significantly wider range of tissues with detectable p16 protein ([12–15,24–26], and this study). Whether inter-species and/or age-related differences [6,12–15,24], or possibly posttranscriptional control mechanisms affecting the protein but not mRNA levels, can explain such significantly different findings remains to be established. Apart from the unexpectedly restricted expression of p19, it appeared that most human tissues express concomitantly two or more members of the INK4 family, possibly providing overlapping functions critical for tissue homeostasis. The concept of at least partly redundant roles in many cell types is also consistent with the limited phenotypes of gene knock-out mice selectively deprived of only one member of the INK4 family [26–29].

3.4. Dynamic expression patterns of the INK4 proteins in differentiating teratocarcinoma cells

The CKIs of the Kip/Cip family have been widely implicated in the switch between proliferation and differentiation [1–4], and recent limited evidence from *in situ* hybridization of mouse neural tissues [14], and *in vitro* differentiation models of muscle [12,15] and hematopoietic cells [25] indicate that the INK4 proteins may also contribute to commitment or maintenance of differentiation, at least in some lineages. Our immunohistochemical analysis showed widespread expression of p18, and detection of some of the INK4 proteins in non-proliferating, terminally differentiated cells and tissues. These results appeared consistent with the potential involvement of the INK4 proteins in differentiation, including for instance the staining of p18 and p15 in adult human muscle, neural ganglia, and some differentiated epithelial tissues. To further elucidate the emerging relationship between expression of the INK4 inhibitors and cell differentiation in a dynamic model system, we explored the commonly used human teratocarcinoma cell line NT2/D1 [9,19], which can be induced to differentiate along the neuronal lineage. Following the established protocols [19], we exposed exponentially growing NT2/D1 cells to retinoic acid and followed their gradual cessation of proliferation, and the eventual emergence of differentiated, quiescent neuronal cells with extended neurites and formation of ganglion-like structures ([9,19], and data not shown). A decisive proliferative change became apparent towards the end of the second week of the induction period, accompanied by disappearance of cyclin B1, induction of Cip/Kip inhibitors such as p21, and the lack of DNA replication (Fig. 4 and data

not shown). Time course immunoblotting analyses of the four INK4 proteins showed reproducible, dynamic patterns characteristic for each family member. Thus, the abundance of p18 remained relatively high and virtually constant throughout the 6-week differentiation period, while p15 was lacking in the exponentially growing cells and became induced very early (at day 1–2 of retinoic acid treatment), and the p15 protein acquired a characteristic double band appearance in the third week of induction (Fig. 4). Unexpectedly, p19 was transiently elevated between days 10 and 18 of the treatment, while p16 remained undetectable for several weeks, and only became induced in the final stages of the experiment, some 3 weeks after the cells became quiescent (Fig. 4). These surprisingly dynamic and complex patterns of the INK4 proteins suggest potential involvement of at least p15 and p19 in the gradual transition from proliferating to quiescent, differentiated cells, and possibly some role of p16 in the terminal differentiation in the NT2/D1 model system. Considered from a more general perspective, these results emphasize the critical impact of the fourth dimension of all biological systems, namely the time-dependent, dynamic patterns of the INK4 proteins during development and tissue renewal. Indeed, the time-dependent expression/accumulation might also account for the puzzling loss of p16 in many human tumors [4–7], in contrast to only rare examples of cancer-associated aberrations of the other, structurally similar INK4 inhibitors [4–9]. Thus, the p16 mRNA and protein gradually accumulate with increasing cell age [6,7], and increase more acutely in response to DNA damage or some oncogenic stimuli [6,7,30], and it may be these timely safeguard responses which distinguish p16 as a guardian of cellular proliferation, and a prominent tumor suppressor.

Acknowledgements: We would like to thank A.A. Kjerulff, Christer Wernstedt, and I.B. Olsen for technical assistance, and Drs. P. Andrews, D. Beach, G. Evan, G. Peters, S. Skjölde Jensen, and A. Wino for providing important reagents. This work was supported by the Danish Cancer Society, the Danish Medical Research Council, the Danish Biotechnology Programme, and the Nordic Cancer Union.

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